Exhibit A

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Serum Interleukin-2-Receptor in Rheumatoid Arthritis: A Prognostic Indicator of Disease Activity?

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Interleukin-2 (IL-2) is an important growth factor for T lymphocytes. Its effects are mediated by cell surface receptors (IL-2 R) expressed on activated T cells. Receptor protein can be shed from cell membranes and the soluble form (sIL-2 R) is detectable by enzyme linked immunosorbent assay (ELISA). We have studied serial levels of sIL-2 R in the sera of patients with rheumatoid arthritis (RA). In 13 patients with active disease, the mean serum level of sIL-2 R was raised compared to age-matched healthy controls. In 48 samples taken at different times from 13 patients, serum sIL-2 R correlated significantly with Ritchie joint index, duration of early morning stiffness, patient pain score, physician's assessment, erythrocyte sedimentation rate (ESR) and platelet count.

In individual patients, serial sIL-2 R serum levels fell with treatment preceding clinical improvement. In four patients where serum sIL-2 R levels fell and clinical improvement occurred, subsequent spontaneous increases of serum sIL-2 R level preceded increased clinical disease activity by up to 2 weeks.

Serum sIL-2 R level in RA probably reflects activation of underlying immunopathogenic mechanisms and appears to be an excellent monitor of clinical disease activity. More importantly, a rising level may also predict exacerbation of disease activity.

Introduction

Activated T lymphocytes produce interleukin-2 (IL-2) and specific cell surface receptors for this important T-cell growth factor [1]. Human IL-2 receptors (IL-2 R) are composed of at least two different polypeptide chains of 55 and 75 kD. Recently a third chain has been described [2]. Each chain alone will bind IL-2 with, respectively, low and intermediate affinities, while the combination of both, forms a high affinity receptor (Kd 10^{-11}M) [3].

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The smaller peptide also known as the Tac protein, is inducible, and as well as being expressed on the surface of activated T cells is released in a soluble form (sIL-2 R) [4]. The rate of release of this soluble protein is proportional to the number of molecules expressed on the cell surface and any cell with surface Tac protein appears capable of releasing sIL-2 R [4]. Although 10 Kd smaller than the cell surface chain (mw 55 Kd), sIL-2 R retains the ability to bind IL-2 [5] with a similar affinity as the cell surface protein [6]. IL-2 R production occurs mainly (but not exclusively) in activated T cells so it might be predicted that raised levels of the soluble form would be found in immune-mediated diseases.

We have found previously that levels of sIL-2 R in sera and synovial fluid exudates from patients with rheumatoid arthritis (RA) were higher than in patients with osteoarthritis and age-matched healthy controls [7]. We also found evidence of immunoregulatory function (down-regulation of *in vitro* cell responses to IL-2) mediated by sIL-2 R [7]. The likely source of this sIL-2 R in RA was shown to be the synovium itself rather than circulating peripheral blood mononuclear cells. We have now performed a serial study of 13 patients with active RA to test the idea that serum levels of sIL-2 R might be related to changes in clinical disease activity.

Methods

Patients

Thirteen patients (seven female) admitted to hospital with active classical or definite RA according to the American Rheumatism Association criteria [8], aged 29–68 (mean 51), were investigated. Eight patients had circulating rheumatoid factor (seropositive), and five had no circulating rheumatoid factor (seronegative). Active disease was defined as: six or more swollen joints, early morning stiffness (EMS) >60 min, Ritchie articular index >15 [9], and erythrocyte sedimentation rate (ESR-Westergren) >40 mm/h. All of the patients were taking non-steroidal anti-inflammatory drugs (NSAIDs) on admission to hospital and during the study. They were all treated with initial bedrest and intra-articular steroid injections and nine were given a 3-week course of daily ACTH injections. Nine patients were started on remission-inducing drugs during the period of study (see Table 1). Sixteen age-matched healthy individuals served as controls. A single patient admitted to hospital with adult-onset Still's disease was also studied.

Clinical methods

Clinical assessment of disease activity was performed by the same physician at 3-day intervals (at least four tests per patient). The following standardised measures were used: Ritchie articular index, duration of morning stiffness (min), patient's pain score (zero representing 'no pain' and 10 representing 'worst possible pain'), physician's global assessment of disease activity, (zero representing 'no activity' and 10 representing 'maximum activity'). 'Onset of clinical improvement' was defined as the day when the Ritchie articular index was below 10, ESR <20 mm/h and EMS <30 min.

Table 1. *Changes in serum sIL-2 R levels in rheumatoid arthritis patients during hospital admission*

	Serum sIL-2 R (U/ml)			
Patient	On admission	Onset of clinical improvement[1]	At discharge	Drug therapy[2]
1	1200	520	400	ACTH
2	1050	510	430	ACTH/S
3	1355	565	NA[3]	ACTH/OG
4	540	250	240	ACTH/OG
5	1240	580	400	ACTH
6	1970	840	480	—
7	720	260	463	ACTH/OG
8	1230	760	930	—
9	570	380	210	ACTH/OG
10	700	530	520	H
11	600	320	420	S
12	1225	920	1370	ACTH/G/P
13	1250	480	NA	ACTH/S
14[4]	5000	1500	500	P

[1]Defined by Ritchie articular index < 10; ESR < 20; duration of morning stiffness < 30 min.
[2]All patients received NSAIDs and intra-articular methylprednisolone injections to large joints (up to three) with active synovitis. ACTH, adrenocorticotrophin; S, salazopyrin; OG, oral gold; H, hydroxychloroquine; G, intramuscular gold; P, prednisolone.
[3]Not available.
[4]Adult-onset Still's disease.

Laboratory methods

Concurrent laboratory assessment was performed by taking blood for haematology tests included in the normal management of RA patients: haemoglobin concentration (Hb), total white cell count (WCC), platelet count (plt), and ESR. A serum sample was separated and stored at −50°C for subsequent sIL-2 R assay. Tests for sIL-2 R were performed on coded samples by individuals who had not been involved in previous clinical evaluations.

ELISA for soluble interleukin-2-receptor

To assay sIL-2 R in patients' sera an ELISA (T Cell Sciences Inc., Biological Industries Ltd, Cumbernauld, Scotland, UK) using two non-competing murine monoclonal antibodies to the Tac protein of the human IL-2 R was used as described previously [7]. Units of sIL-2 R were calculated from a standard curve constructed using a supernatant from phytohaemagglutinin-stimulated peripheral blood mononuclear cells. Samples were assayed in duplicate on at least two separate occasions and inter-assay variability was 8±3%. Normal sera or sera from patients caused no interference in the ELISA as determined by detection of added standard sIL-2 R.

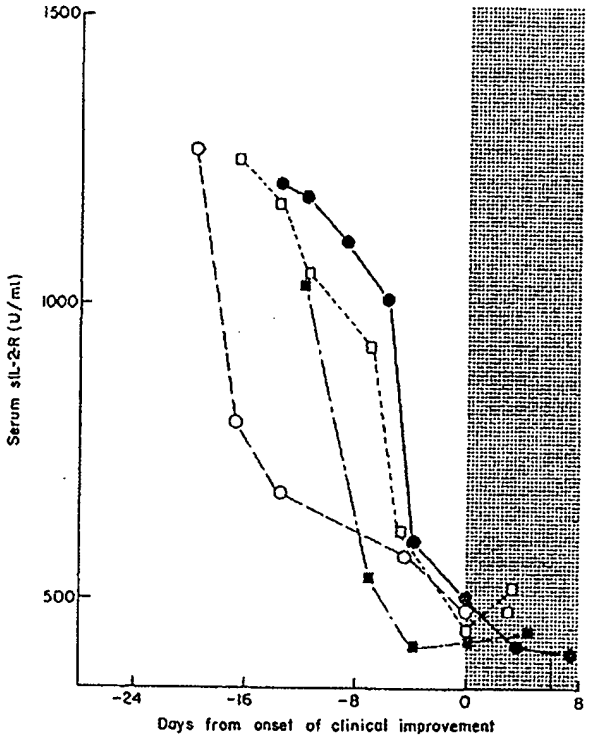

Figure 1. Serial sIL-2 R serum levels in four patients with active rheumatoid arthritis: ●——●, patient 1; ■——■, patient 2; O---O, patient 5; □·····□, patient 13. 'Onset of clinical improvement' is defined in methods section. The first sIL-2 R measurement for each patient was on the day of admission to hospital. Mean (±SEM) serum sIL-2 R concentration in 16 healthy age-matched controls was 470±35 U/ml. Shaded region indicates onset of clinical improvement.

Statistical methods

Comparisons of means were tested with Student's *t*-test (double tailed) and correlation assessed by Spearman's Rank Correlation Coefficient.

Results

Serial sIL-2 R levels and clinical improvement

Initial serum sIL-2 R level in 13 patients admitted to hospital with active RA was 1050±109 U/ml (mean±SEM). This was significantly higher than in 16 age- and sex-matched healthy controls (470±35 U/ml; $P<0.001$). There was no significant difference in serum sIL-2 R level between eight seropositive patients and five seronegative patients, 1012±107 *vs* 1050±184.

In each patient a marked drop in serum sIL-2 R level occurred following treatment and preceded observed clinical improvement. In two cases where only NSAIDs and intra-articular steroids were used, similar sIL-2 R changes occurred. Figure 1 shows

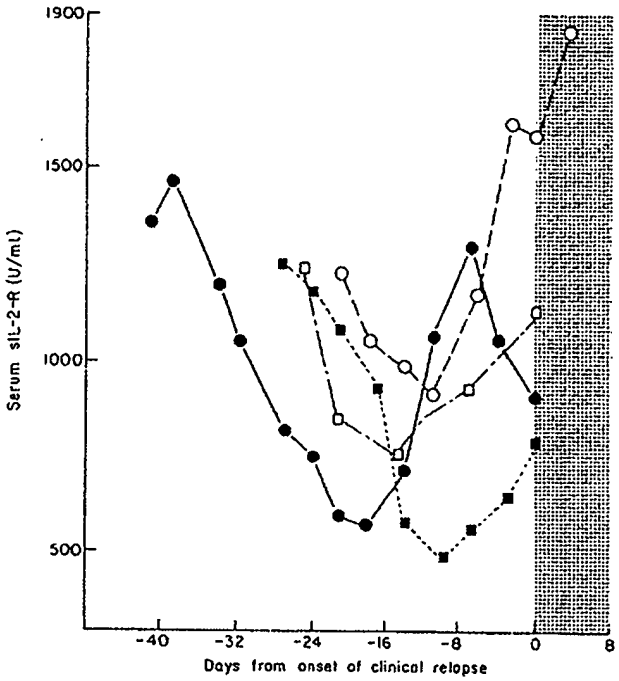

Figure 2. Serial sIL-2 R serum levels in four RA patients whose levels initially fell preceeding temporary clinical improvement and subsequently increased again preceeding 'clinical relapse' (defined by: increase in Ritchie index > 10; increase in ESR > 20 mm/h; increasing EMS > 30 min). ●——●, patient 3; □——□, patient 8; ○- - -○, patient 12; ■·····■, patient 13.

the serial serum sIL-2 R levels in four of the 13 RA patients. Similar results were obtained from the other patients and in nine of these 13 patients this fall in serum sIL-2 R level was maintained until discharge from hospital when serum sIL-2 R levels were not significantly different from the age-matched control population (Table 1).

Serial sIL-2 R levels and clinical exacerbation

In four patients the serum sIL-2 R level increased again following the period of clinical improvement (Figure 2). In patient three, the level rose progressively from 565 U/ml during clinical remission to 1,300 U/ml over an 11-day period. Subsequently, a generalised exacerbation of rheumatoid symptoms occurred 7 days after this second rise in sIL-2 R level had reached a maximum. This prolonged the patient's hospital admission. Similarly the serum sIL-2 R level in patient eight had increased from 760 U/ml at the time of clinical improvement to 930 U/ml at the time of discharge. The patient initially remained clinically stable at home but a generalised exacerbation of rheumatoid symptoms occurred after 7 days and required treatment with oral steroids. At this stage, serum sIL-2 R level was measured as 1,120 U/ml. The serum sIL-2 R level in patient 12 had fallen to 920 U/ml at the time of clinical remission but subsequently began to rise. The patient had a marked exacerbation of

symptoms starting 6 days after this rise in the serum sIL-2 R and was subsequently treated with oral steroids. In patient 13, the serum sIL-2 R level was 480 U/ml during clinical remission. Serum sIL-2 R levels then began to rise reaching 780 U/ml at the time of clinical relapse, which occurred 7 days after the first rise in sIL-2 R had been detected.

Still's disease

The highest serum sIL-2 R levels were observed in the one patient with adult Still's disease (5,000 U/ml). This patient presented with a classical multisystem illness including high fever, skin rash, generalised lymphadenopathy, hepatosplenomegaly, pharyngitis and polyarthritis without evidence of infection or disease-associated auto-antibodies. At the time of discharge, when the patient was clinically well but still receiving high-dose steroids, the sIL-2 R level was in the range seen in healthy controls.

Correlations of sIL-2 R serum levels with measures of disease activity

Overall, serum sIL-2 R levels were found to correlate significantly with several clinical measures of disease activity. In total 48 observations were made in the 14 patients over a mean study duration of 25 days. Serum sIL-2 R correlated significantly with: Ritchie articular index ($r = +0.542$, $P < 0.001$), duration of morning stiffness ($r = +0.617$, $P < 0.001$), patient pain score ($r = +0.608$, $P < 0.001$), and physician's global score ($r = +0.616$, $P < 0.001$) (Figure 3).

Concurrent sIL-2 R and haematological laboratory tests were performed on 50 occasions in this group of patients. ESR and platelet count correlated significantly with sIL-2 R ($r = +0.665$, $P < 0.001$; $r = +0.481$, $P < 0.001$) respectively (Figure 4).

There was no significant correlation between sIL-2 R and haemoglobin concentration or total white cell count.

Discussion

The binding of IL-2 to its cell-surface receptor (IL-2 R) leads to clonal expansion of antigen-triggered T-cell subsets [10] and is probably an essential event in the generation of most immune responses. The induction of IL-2 and its receptor are therefore central to immune activation. Since IL-2 R is shed from cells in proportion to its level of surface expression, the detection of these molecules provides an opportunity to follow immune activation *in vivo*. Previously we have shown that in RA serum sIL-2 R is probably derived from the synovium itself rather than from peripheral blood mononuclear cells [7]. This supports the idea that the serum content of receptor protein reflects immunopathogenic mechanisms within the joint.

In the present study of 13 patients admitted to hospital with active RA and one patient with an acute presentation of adult-onset Still's disease, we found that serum sIL-2 R levels correlated significantly with several conventional clinical and laboratory measures of disease activity. These included duration of early morning stiffness, Ritchie articular index, patient pain score and the physician's global assessment of

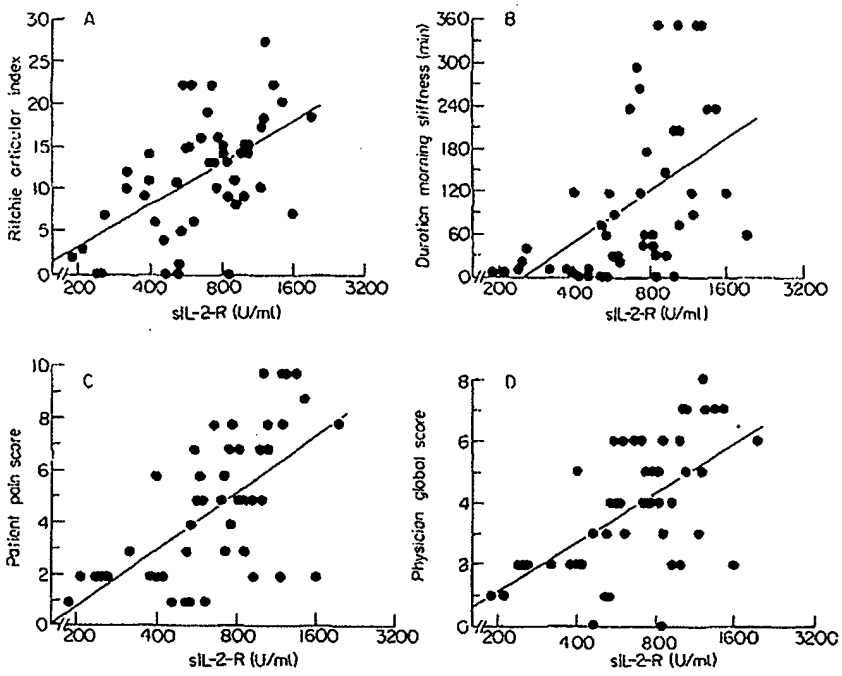

Figure 3. Correlation between measures of clinical disease activity and serum sIL-2 R in whole group (3–4 measurements per patient at different times). A, sIL-2 R *vs* Ritchie score, $r = +0.542$ $P < 0.001$; B, sIL-2 R *vs* duration of morning stiffness, $r = +0.617$, $P < 0.001$; C, sIL-2 R *vs* patient pain score, $r = +0.608$, $P < 0.001$; D, sIL-2 R *vs* physician's global score, $r = +0.616$, $P < 0.001$.

disease activity. Of the laboratory measurements, only ESR and platelet count were significantly correlated with serum sIL-2 R.

On admission to hospital all patients had significantly elevated serum sIL-2 R levels compared to age-matched, healthy controls. Following therapy, a marked fall in serum sIL-2 R level occurred in each patient and preceeded clinical improvement by 4–8 days. This decrease in serum sIL-2 R level occurred in two patients where only NSAIDs and intra-articular steroids had been given. All patients received intra-articular steroid (methyl prednisolone) and nine were treated with intramuscular ACTH. It is known that corticosteroids can inhibit transcription of the IL-2-receptor Tac gene in human blood mononuclear cells [11].

In four of the 13 patients with RA, following initial clinical improvement, serum sIL-2 R levels again began to rise. In each of these patients a subsequent exacerbation of rheumatoid symptoms occurred. This second rise in serum sIL-2 R level began 6–13 d prior to the observed clinical deterioration. These results indicate that in individual patients, rising levels of serum sIL-2 R might predict increased clinical disease activity in inflammatory arthritis. It is also possible that, in some circumstances, clinical exacerbation may be avoided by early immunosuppressive therapy started at the time of sIL-2 R increase.

It is noteworthy that one patient developed a bacterial urinary tract infection during the course of the study and received antibiotics. This was not associated with

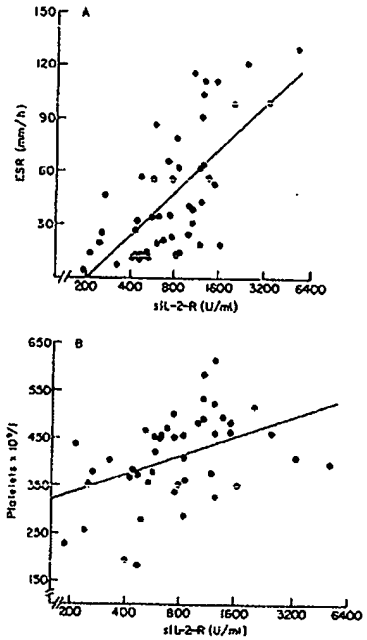

Figure 4. Correlation between laboratory measures of disease activity and concurrent serum sIL-2 R levels in whole group (at least three tests per patient at different times). A, sIL-2 R *vs* ESR, $r = +0.665$, $P < 0.001$; *B*, sIL-2 R *vs* platelets, $r = +0.481$, $P < 0.001$.

increasing sIL-2 R levels which continued to fall over this period. Increased sIL-2 R levels are not, of course, specific for RA but occur in other immune-mediated conditions such as renal graft rejection [12] and in haematological malignancy [13]. In previous work we have found that, beyond its usefulness as a measure of immune activity, sIL-2 R may have functional significance by competing with cell-associated IL-2 R for available IL-2. This competitive inhibition of IL-2 may well contribute to the well-documented defects [14] in cell-mediated immunity that occur in RA and other chronic inflammatory diseases. Whether this immune defect can itself be pathogenic remains to be determined.

Traditional laboratory indices of disease activity in inflammatory arthritis are thought to reflect the acute phase response rather than measure the immunopathogenic mechanisms underlying clinical disease. The T cell is the predominent cell type in the rheumatoid synovium and serum sIL-2 R seems to derive from the synovium as a consequence of T-cell activation. We have shown in this longitudinal study that serum sIL-2 R is related to disease activity in RA and may well be a relatively simple measure of immunopathogenic activity. A fall in the serum sIL-2 R level in each of our patients preceded clinical improvement and in four patients a rise predicted subsequent disease exacerbation.

This soluble receptor protein offers a new measure of clinical disease activity and may be very useful in clinical trials of treatments for rheumatoid arthritis. Increasing sIL-2 R levels might even predict disease exacerbation and allow earlier intervention.

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